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Introduction

This proposal aims to establish human neural progenitor cell lines releasing GDNF for potential therapy for Parkinson's disease. One section of this year's update can be found in Appendix one which is a near final version of a paper about to be submitted to the journal Gene Therapy (Behrstock et al, 2005). This pre-publication now includes a completed transplant study (last year we just had pilot data) showing robust survival and release of GDNF from human neural progenitor cells. During the first year very rapid progress was made with regard to infection of the cells with an inducible GDNF lentiviral constructs. However, following the first transplants using these cells it was clear that the dual vector system, while efficient *in vitro* was not working well *in vivo* following transplantation. This led us back to the basic science of vector infection of the human progenitors and attempts to increase efficiency of infection. To reach this goal we have performed extensive analysis on the basic infection protocols for human neural progenitor cells using the same viral backbone as the inducible system but a constitutive release system in order to establish optimum protocols. This new data is included below and allowed us to proceed further with the inducible constructs. During the past year our collaborators in Switzerland have been working to develop more efficient single lentiviral inducible vectors. Updates to vector design include using only a single vector which reduces the need to do double infections of cells. Also included in this new construct is the KRAB system which has a humanized element that which is far less likely to induce immune rejection in human patients (one of the concerns with the old tet regulated systems). These vectors have now been tested by us and found to be far more efficient and reliable *in vitro* than the dual constructs used previously. Based on these new results we are now ready to proceed with extensive new transplant studies in rodents to test the new single tet regulated lentiviral vector.

Our imaging studies have progress well. We now have a much more efficient marker for dopamine neurons and have established a new stereotaxic frame system for the simultaneous analysis of 4 rats for PET. This system is now ready for the new stem cell transplants and carrying out the experiments outlined in year three which include PET, GDNF releasing human progenitors and functional studies. Our pilot monkey project funded through a contract with Jeff Kordower at Rush University is now complete and is included in the publication outlined in Appendix one. Here three aged monkeys received transplants of human neural progenitor cells. One died during the course of the study. Out of the other two one was found to have a significant transplant that released large amounts of GDNF based on staining of the brain. It is possible that in this xenograft model the cells rejected in one of these animals. We are currently we are performing immunostaining to look for rejecting cells. The funding for this pilot study is now complete but it has allowed us to attract other funding which is enabling a continuation of this project using younger monkeys with MPTP lesions. This is in collaboration with Dr. Marina Emborg who has just moved to the University of Wisconsin.

During the course of last year Dr. Soshana Behrstock left the laboratory following her three year post doctoral training to work at a local biotech company Promega. She has now been replaced by Dr. Allison Ebert who has recently completed her PhD with Dr. Marty Bohn, a world leader in GDNF research and inducible vector systems. Dr. Ebert is ideally qualified to continue the research and her CV is attached as Appendix 2.

Body

Task 1. To produce rat and monkey neural stem cells which secrete GDNF under an inducible promoter.

- a. Assess and optimize GDNF release from rat and monkey neurospheres using lentiviral vectors (Months 1-18).**

As noted in last year's progress report, we used human neural progenitor cells (hNPCs) rather than pursuing rat and monkey progenitor cells because of technical issues with being able to expand the monkey cells. Moreover, last year's report showed that much of *Task 1* was completed. Specifically, we found that hNPC infected with a dual lentiviral system expressing GDNF by an inducible promoter could be grown for long periods of time and that the GDNF could be regulated in vitro. However, when we transplanted these cells into a rat model of PD to address *Task 2c*, GDNF gene expression was not

regulated by DOX. In addition we found some variability between cultures with regard to overall expression. While some regulated very well, others were hard to switch on and off over longer periods of time. For this reason we went back to optimizing the basic infection protocols and establishing FACS

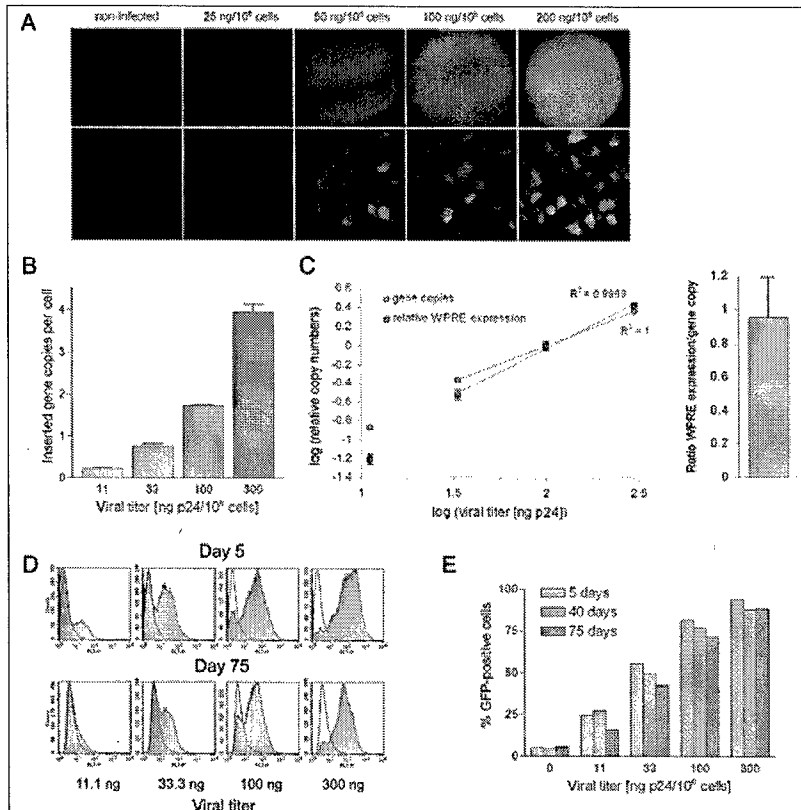


Fig. 1. Optimization of hNPC neurosphere infection using the SIN-PGK-GFP-WPRE lentivirus (A) GFP fluorescence in whole neurospheres and dissociated cells is shown for increasing viral titers. (B) Number of integrated gene copies one month after infection determined by qPCR (WPRE versus albumin). (C) Comparison of relative transcript and genomic transgene copy abundance as determined by qPCR. (D,E) Flow cytometric determination of the proportion of GFP-positive hNPCs during expansion in culture.

analysis of the progenitors as outlined in the grant. A summary of these results are presented below. This data is currently being written up as a methods paper for publication.

Optimization of hNPC transduction using a lentiviral vector for constitutive GFP expression

In order to maximize accessibility of the targeted cells to the viral vector, hNPC neurospheres were dissociated and then exposed in suspension for 24 hrs to a lentivirus designed for constitutive green fluorescent protein (GFP) expression under the control of the PGK promoter. Transcript stability was enhanced by incorporation of the posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE or WHV) (Zufferey et al., 1999; Deglon et al., 2000). One day after infection, cells spontaneously reformed spheres in culture, allowing their rapid expansion under standard culture conditions. Four days after infection with increasing viral concentrations, GFP expression was analyzed by microscopy (Fig. 1A). One month post-infection, the number of transgene copies integrated in the genome was determined by quantitative PCR (qPCR), by comparing the amplification of the WPRE sequence with an

albumin sequence (2 copies/genome) (Fig. 1B). At the same time, the abundance of transgene transcripts was determined by comparing the amplification of the reverse transcribed WPRE sequence with the β -actin transcript as a reference (Fig. 1C). We observed similar, linear increases in integrated transgene genomic copies and transcripts in response to increasing viral loads. Remarkably, the relative ratio of transcript/gene copy remained similar for the viral doses of 33, 100 and 300 ng of p24 (bar graph on Fig. 1C, expressed as average ratio \pm standard deviation). In parallel, GFP protein expression was determined by flow cytometry on day 5, 40 and 75 after infection, showing a reproducible stability in the proportion of transgenic hNPC during expansion (Fig. 1D,E) and demonstrating that >80% of GFP-expressing cells could be obtained with minimal cytotoxic effects

Maintenance of GFP expression in differentiated cells derived from transgenic hNPC

Because of the developmentally controlled genomic remodeling of transcriptional activity, transgene expression might be lost during differentiation. To address this question, the GFP-expressing hNPC populations described above were differentiated after 75 days of expansion. Spheres were plated on poly-L-lysine/laminin for 1 week in the absence of growth factors, and then kept for 2 additional weeks in presence of 1% fetal bovine serum. At this time, two distinct populations could be observed, one with a neuronal morphology and the other with an astrocytic morphology. Cells were dissociated, immunostained for β -III-tubulin and GFAP (glial acidic fibrillary protein), and analyzed by flow cytometry (Fig. 2A). A small-sized sub-population could be distinguished, positively immunostained for β -III-tubulin and negative for GFAP, almost purely neuronal (R2). Larger cells were essentially GFAP-positive, denoting either astrocytes or progenitors, although a small population of large-sized neurons also appears to be present (R1). GFP-infected differentiated hNPC were also analyzed by flow cytometry, and the two populations described above were gated according to size and granularity. GFP-positive cells were quantified, showing only a slight decrease in the percentage of positive cells in each of these two populations after three weeks of differentiation (Fig. 2B,C; compare Fig. 2C to 3E). To verify that transgene integration did not alter the differentiation ability of the hNPC populations, GFP-expressing spheres were plated for 8 days in the absence of growth factors on laminin substrate. The cells were then dissociated, re plated and the percentage of β -III-tubulin-positive (neuronal) and GFAP-positive (non-neuronal) cells determined by immunocytochemistry and fluorescence microscopy (Fig. 2D). There were no significant changes in the percentages of cells positive for these two early markers across the different hNPC populations.

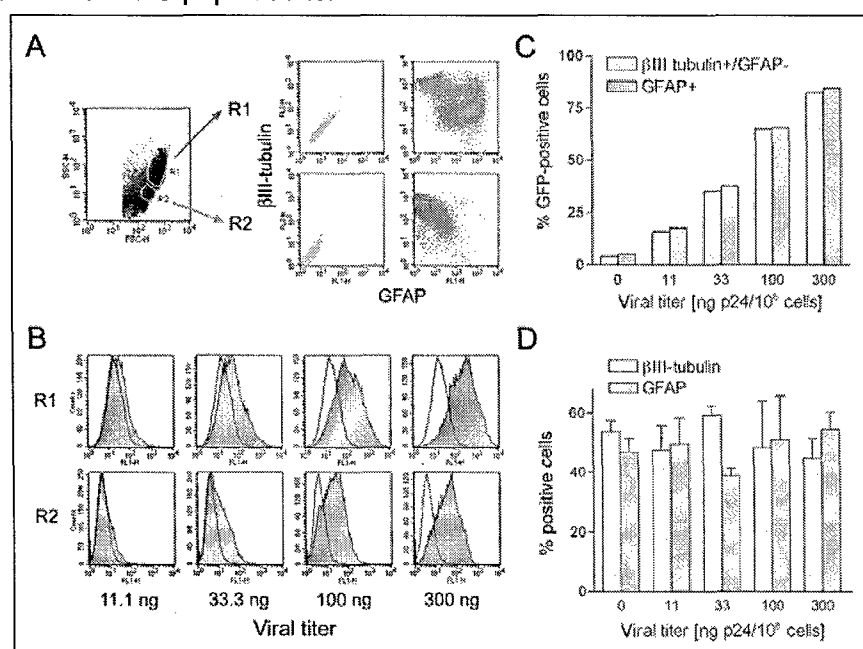


Fig. 2. Differentiation of GFP-transgenic hNPCs (A) Flow cytometry of hNPC differentiated for 3 weeks. Cells were immunostained for β -III-tubulin and GFAP. (B) Flow cytometry analysis for GFP in the R1 and R2 sub-populations. (C) Percentages of GFP positive cells in the differentiated R1 and R2 sub populations. (D) GFP transgene integration does not affect the differentiation of hNPC populations: percentages of β -III-tubulin and GFAP positive cells after 8 days of differentiation.

GDNF production by lenti-infected hNPC

We next established whether infection with lenti^{GDNF} using identical methods to GFP as described above would result in stable lines producing this protein. Our data shows that GDNF is reliably produced in hNPC for up to 14 weeks of expansion and continues to be expressed at similar levels 50 days following differentiation into astrocytes and neurons (Fig. 3, A,B,C). This was one of the specific goals of this grant. GDNF expression in hNPC had no effect on the capacity of cells to differentiate into neurons and astrocytes based on marker studies following differentiation (Fig. 3D).

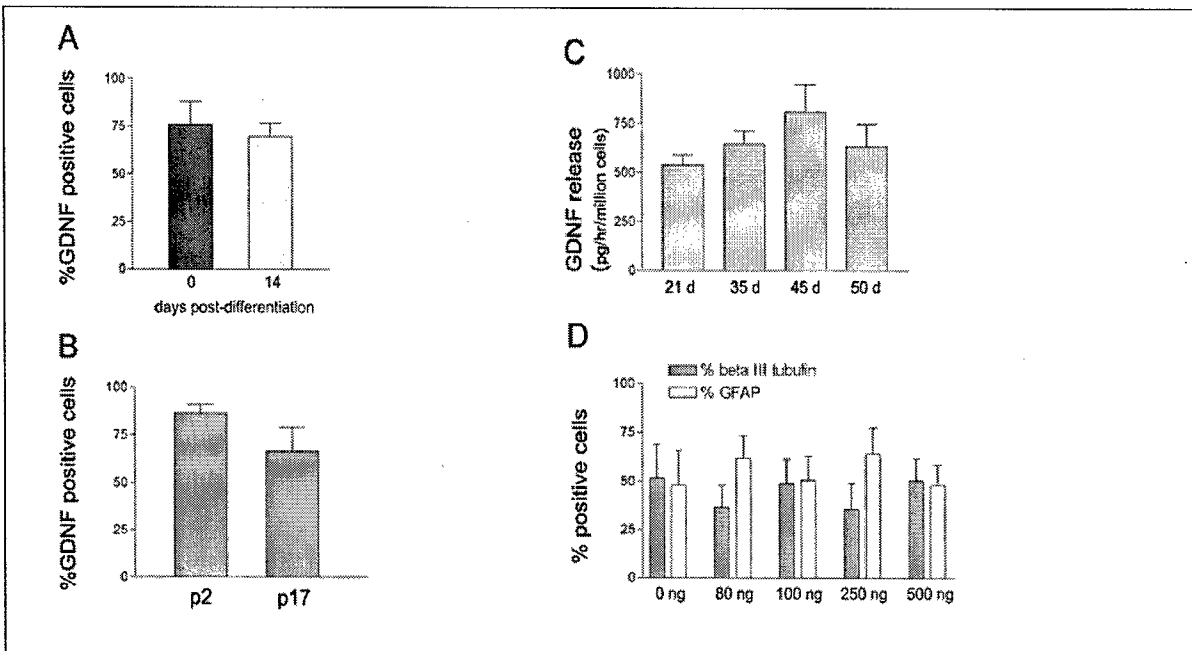


Figure 3. GDNF expressing hNPCs do not down-regulate the transgene after differentiation or with time in culture, nor does expression affect the differentiation potential of the cells. A) Quantification of immunocytochemistry for number of cells expressing GDNF before differentiation (0 days post-differentiation) or after two weeks of growth factor withdrawal (14 days post-differentiation). B) Number of cells expressing GDNF in growth factor stimulated neurosphere cultures early after infection (p2) or after 12 weeks in growth factor stimulated neurosphere culture (p17). C) GDNF release as measured by ELISA from differentiated cells maintained in the absence of growth factors for up to 50 days. D) Quantification of numbers of neurons (beta III tubulin positive-dark bars) or astrocytes (GFAP positive-open bars) produced by hNPC populations either uninfected (0 ng) or infected with increasing titers of GDNF transgene containing virus (80, 100, 250 or 500 ng p24/million cells) then differentiated for 14 days in the absence of growth factors.

Characterization of hGDNF produced by hNPC

Preliminary characterization of GDNF from hNSCs suggests that it is fundamentally different from GDNF which is produced in *E. coli*. This difference is due to the fact that, unlike *E. coli*, mammalian cell lines (e.g., NSC) are capable of performing post-translational modifications such as glycosylation. Indeed, initial characterization of GDNF from the murine B9 cell line indicated that GDNF from mammalian cells is highly glycosylated. This glycosylation may have a profound effect on the immunogenicity of the

protein as well as the bioactivity and in-vivo stability. Comparison with rhGDNF using SDS-PAGE/western blotting demonstrated that GDNF from NSC exhibits a higher apparent molecular weight relative to E. coli-derived GDNF and exhibits heterogeneity as would be expected for a glycosylated protein (See Figure 1 in Appendix 1). Deglycosylation studies will be performed to verify the difference in glycosylation between rhGDNF produced in E. coli (Amgen) and the transgenic protein made by hNSC.

A new single vector inducible system.

We have modified our infection protocol for delivering transgene to hNSC and now can carefully control for equal numbers of insertions within separate populations (see Figures above). This allows direct comparison of GDNF promoter expression systems. The tet-off system involves dual virus delivery of constitutively expressed tTA transactivator on one virus and the GDNF transgene under the control of the tet-regulated promoter on the second virus. This system has been used extensively in the lab for *in vitro* and *in vivo* expression studies. However, we have found poor regulation of gene expression *in vivo* using this construct and never achieved 100% shut off of the gene *in vitro*. Therefore, we have explored the use of a new vector system which should be more reliable based on the literature. This is also a tet-on system which we feel is now more relevant clinically given some of the issues raised by AMGEN regarding GDNF safety in monkeys.

The new tet-on system is based on a tet-regulated tTRK transcriptional repressor and is delivered on a single virus expressing GDNF from a regulated promoter and the transcriptional repressor via an internal ribosomal entry site (IRES) (Fig. 4)

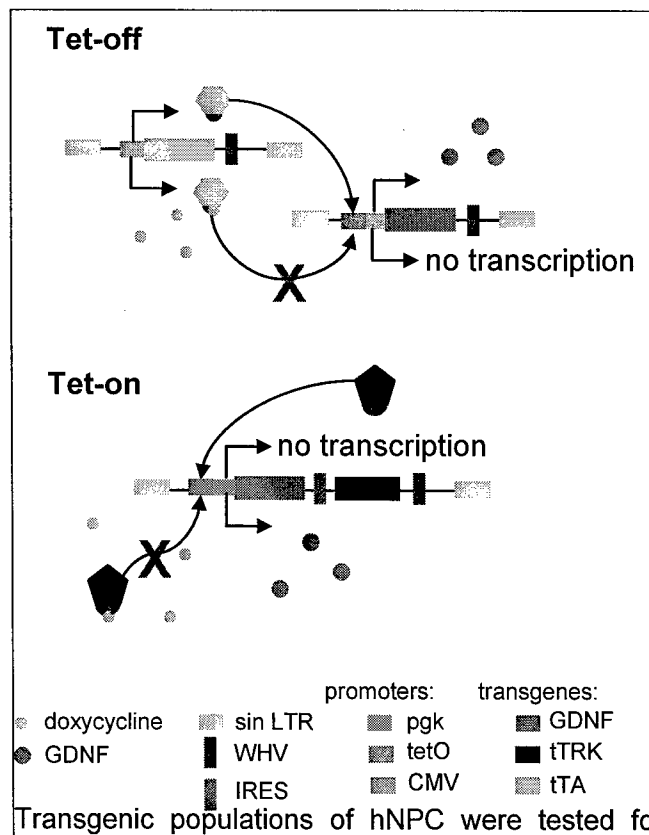


Fig. 4. Schematic showing how the two virus tet-off system and the single virus tet-on system affect transcription of the GDNF transgene in the presence or absence of the tetracycline analog doxycycline (dox). The transcriptional units shown are representative of the proviral inserted DNA.

Transgenic populations of hNPC were tested for amount of GDNF produced by ELISA. For the tetracycline regulated lines, the promoters were turned on (no dox for the tet-off line and + dox for the tet-

on line). The left panel in Fig 5 below shows the number of proviral insertions determined by qPCR using Taqman assays for either GDNF specific or transgene specific (WHV) probes. The tet-off dual virus delivery system shows slightly less than the expected level of GDNF virus, reflecting the lack of control of integration with two viruses. The constitutive virus clearly produces more GDNF than the regulated viruses. However, the exact dosing of GDNF required for functional effects has yet to be determined.

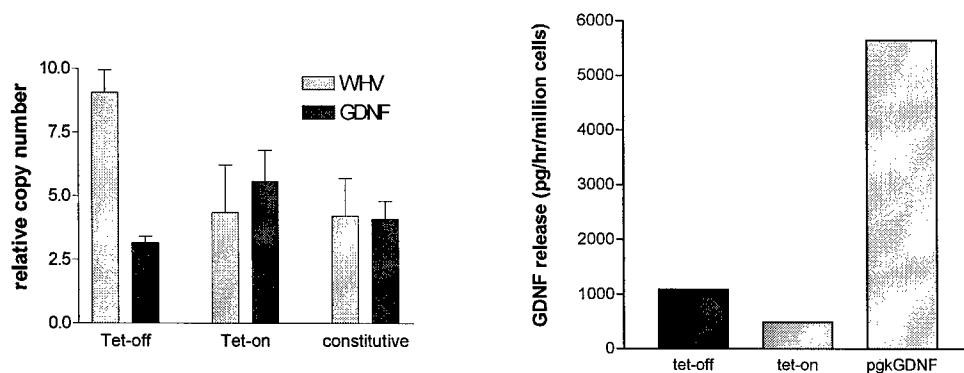


Figure 5. Copy number and GDNF release using different viral vectors

The tet-on system has been analyzed extensively *in vitro* but hNPC infected using this system have not yet been transplanted into animal models. This is one of the aims in year three. It is clear from the analysis presented in figure 5, that co-expressing a regulatory protein impacts on the amount of GDNF produced by transgenic hNPC. In addition, using a repressor-based system further reduces the amount of GDNF produced. However, despite the reduction in amount of GDNF produced, the tet-on system shows significant advantage over the tet-off system with regards to the tightness of the regulation and the ability of the promoter to cycle on and off *in vitro*. Figure 6 shows typical results from experiments turning on and off the promoter in the tet-on lines.

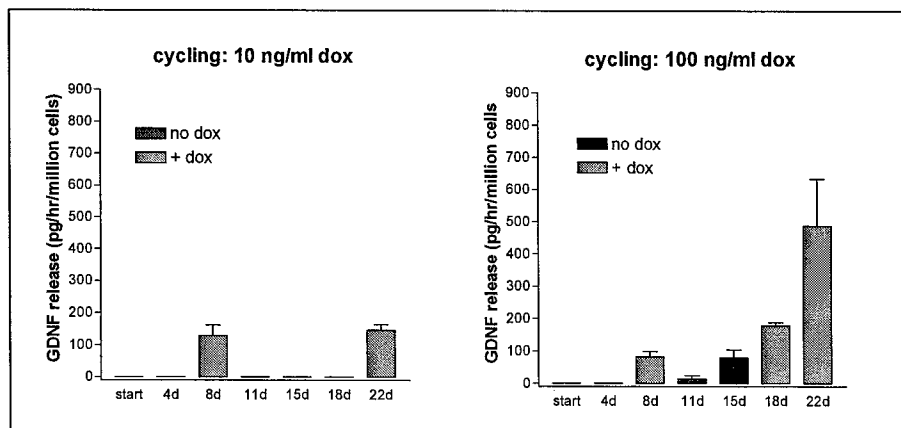


Fig. 6. Response of the tet-on regulated promoter to dox: cells from a culture where the promoter was turned off were maintained in either 10 or 100 ng/ml dox for one week, washed and maintained without dox for one week then maintained in either 10 or 100 ng/ml dox for one week.

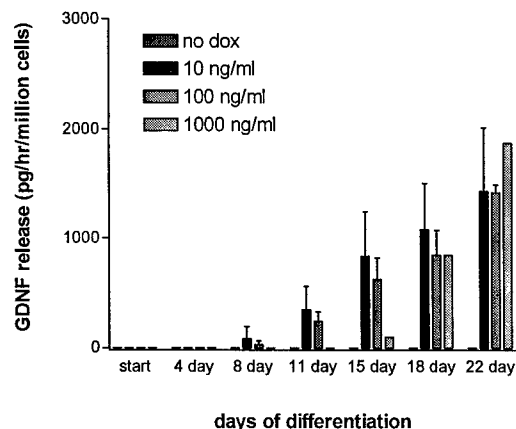


Fig. 7. Response to DOX of inducible single vector system. Same cells plated and maintained in the presence of three orders of magnitude of dox for three weeks. Note increased GDNF release with increasing DOX levels.

The transgenic lines respond equally well to three orders of magnitude of dox (10 ng/ml to 1000 ng/ml) although the ability of the cells to quickly turn on or off the promoter depends on the amount of inducer to which they are exposed (Fig. 7). In addition, we have found it is easier to control the number of proviral insertions as well as the cells' response to dox titration using single virus delivery. Finally, using an IRES to control translation of the regulatory protein reduces the levels of expression of potentially toxic exogenous protein. For the tet-off system, the higher rate of infection produced by the modified infection protocol has resulted in loss of regulation and high background levels of transgene expression after long term exposure to doxycycline.

We have focused recent efforts on the tet-on system because from a clinical standpoint it is more appealing to take a drug to turn the promoter on and because background expression during the off state needs to be negligible to be clinically safe. It remains to be seen whether the amounts of GDNF produced by transgenic lines switched on are therapeutically useful but these studies are now underway.

c. Select and characterize lentiviral clones which express GDNF at high levels following differentiation (Months 18-36).

This task has been completed. Although not clones we have lines of cells with predictable expression levels of GDNF. We are now enhancing infection protocols and establishing regulated release as described in the results above.

d. Produce and optimize new vectors with combinations of GDNF/GFP (retrovirus/AAV) - assess which is most efficient at GDNF production *in vitro* and compare with lentivirus (Months 6 - 48).

Due to the success with the lentiviral constructs all of our efforts have been towards optimizing infection protocols using this delivery system. This is described in some detail in the results above.

Task 2. To protect against toxic cell death in the brain by transplanting GDNF producing stem cells into rodent and primate models of PD.

- a. Perform pilot monkey transplant study with GFP/GDNF retroviral construct using micro-PET and post mortem data to establish survival and possible function of cells (Months 6-18).**

Please refer to Appendix 1 for an update on the monkey transplant studies. Our collaborator Dr. Jeffery Kordower at Rush St. Luke's Medical Center has completed a pilot study showing effective delivery and survival of GDNF expressing hNSC in the caudate of three aged primates. Human neural progenitors survived transplantation in one of the three animals. Moreover, immunohistochemistry for GDNF showed robust GDNF expression corresponding to the transplant site. These primates were not lesioned with MPTP to induce a model of PD, so no functional assays were performed. Dr. Kordower is currently performing more analysis on the tissue sections from the other two animals and we will be able to report this data in the next update.

- b. Assess optimal source and preparation (FACS, pre-differentiation) of rodent neural stem cells for grafting (Months 12-48).**

In task 1a we have described our extensive work on optimizing the infection protocols and FACS analysis. We are in the process of using this information in the transplant studies described below.

- c. Assess whether rodent GDNF secreting stem cells prevent neurotoxic cell death and how this relates to dopamine storage in the brain using micro-PET (Months 18-36)**

These studies are now well under way. As outlined in the proposal, we asked whether GDNF expressing hNSC would provide protection for the dopamine neurons affected by a 6-OHDA lesion. We found that GDNF expressing hNSC were shown to migrate throughout the adult rat striatum and produce robust GDNF expression. These studies are described in detail within Appendix 1 (Behrstock et al, 2005). In short term studies GDNF released by the transplanted cells induced sprouting of host dopamine fibers but this was not seen with wild type cells and increased survival of dopamine neurons in the substantia nigra (Figure 3, Appendix 1). We next assessed behavioral recovery by the amphetamine-induced rotation assay. Amphetamine-induced rotational behavior was tested prior to and following GDNF hNPC transplants. The number of rotations correlates with the severity of the dopamine neuron loss. Rats with the GDNF expressing hNSC showed fewer rotations over a 6 week period. However, because of spontaneous recovery in the un-transplanted rats, which we attribute to daily cyclosporine injections (Matsuura et al., 1997), this recovery trend did not reach significance when compared over the 10 week experiment using ANOVA tests (Appendix 1, Fig. 5). However, there were significantly more dopamine neurons surviving in the substantia nigra of the transplanted when compared to control animals (Appendix 1, Figure 5). We currently revising our lesion protocol to ensure more substantial damage and minimize spontaneous recovery.

We also analyzed whether GDNF expressing hNPC differentiated into various brain cell types, including astrocytes and neurons, or whether they maintained progenitor cell characteristics. Immunohistochemical analysis revealed that over 90% of the hNSC were nestin positive, suggestive of progenitor cells, while less than 5% were GFAP positive, which labels astrocytes (Appendix 1, Fig. 6). Labeling with a human specific neuronal marker did not detect differentiation into neurons. In a more detailed analysis of the transplant it was clear that cells that remained within the graft were GFAP positive – but those that migrated away from the graft expressed nestin at 8 weeks. We feel that over time some of the migrating cells would down regulate nestin and start to express GFAP. Longer term experiments are currently

being planned. The conclusion of this paper is that GDNF delivery using human neural stem cells is possible and has functional effects both in vitro and in vivo.

PET analysis was not performed in the experiment described above as we are still optimizing the procedure. However, we have made great strides in optimizing the PET imaging procedure and are now ready to proceed. This year was marked by both major advances and a major protocol change in the program of studies on the microPET small-animal scanner that are used to document the neurophysiological correlates of brain repair in rats. Because the largest cost involved in the PET scanning is the cost of the labeled tracer, it is not feasible to support a program in which one tracer synthesis yields a study in a single rat. We completed the design and construction of a stereotactic head-holder that allows reproducible rigid positioning of four rats simultaneously. Each position is fitted with the standard bite bar/ear bar combination found in surgical stereotactic units, in addition to conical anesthesia masks, also identical to those found on commercial surgical devices. A pair of technicians can position four rats in less than a half hour.

Another major obstacle that was overcome was tracer administration. The difficulty of performing four tail-vein angiocatheterizations in just a few minutes left us with large likelihoods of at least one failed study out of four. We have tested the feasibility of using intraperitoneal administration, and found it to be safe and reliable. Both this and the positioning device have served to greatly reduce the anesthetic burden suffered by these very valuable treated rats.

Finally, we have changed the tracer of choice for demonstrating the restoration of dopaminergic function in treated animals. The original choice of fluoro-*m*-tyrosine (FMT), a tracer of aromatic amino acid decarboxylase, was based on our extensive prior experience in larger animals and a small number of pilot studies in rats. These early studies have been difficult to reproduce. Library research has yielded that the basal ganglia versus whole brain excess of decarboxylase activity in rats is an order of magnitude smaller than that in primates. We thus developed the synthesis of a tracer compound that hadn't previously been made here, ¹¹C labeled dihydrotetrabenazine (DTBZ). This compound is specific to the transporter protein in the monoamine vesicles found in dopaminergic nerve terminals (VMAT). Pilot data from a partially lesioned animal are shown in Figure 4. The excess of signal in the striatum is specifically and proportionally related to the concentration of VMAT. Increase of the signal in a post-treatment study relative to the pre-treatment baseline can be due only to the increased presence of functioning monoamine vesicles. Because the binding process requires energy and appropriate levels of cofactors, the DTBZ signal is indicative not only of the presence of vesicles, but their presence in a normally functioning environment. Finally, a further advantage of the change of tracer is that the studies are 60 minutes rather than 90 minutes in duration, further reducing the anesthetic burden.

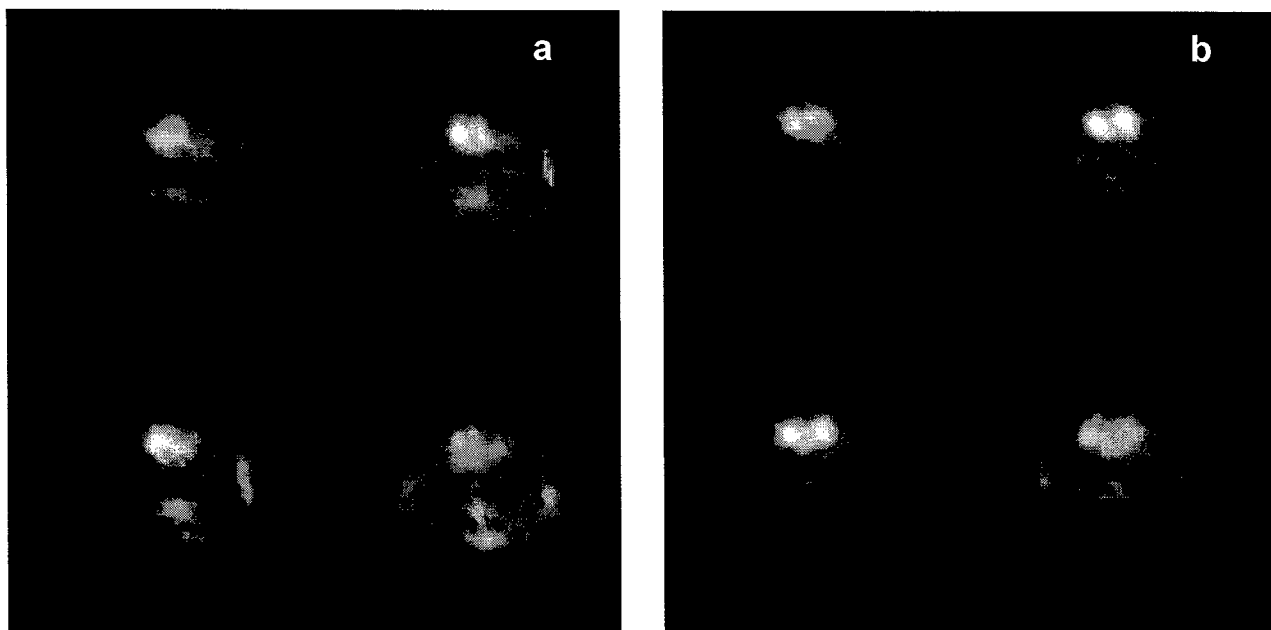


Fig. 8. Coronal slices of PET scans showing vesicular monoamine transporters in rat striatum: (a) partial lesion of right striatum indicated by arrow and (b) control animal. The images show parametric maps of the [^{11}C]DTBZ distribution volume ratio with cerebellum as the reference tissue.

- d. **Prove regulated delivery can be achieved, and establish effects of switching GDNF on and off on dopamine storage in the brain using micro-PET (Months 24- 48).**

These studies have just been initiated.

Key research accomplishments

We have shown for the first time that human neural progenitors can survive and migrate within the rat lesioned brain and release GDNF for up to 10 weeks. This is sufficient to increase the number of dopamine neurons within the substantia nigra. The GDNF released by the cells is glycosylated and distributes evenly within the striatum. Our next generation of inducible vector systems are now ready for in vivo testing in year three. We have also accomplished our goal of having a four rat PET system ready for use in this regulated study to assess the effects of GDNF release from the progenitor cells on

Reportable Outcomes

During the last year we have been focusing on generating the first paper from the data from this grant which is attached as Appendix 1. In addition we have recently published a paper showing survival, integration and differentiation of GDNF secreting cells similar to those described in the current proposal into the spinal cord or rats with a genetic form of ALS (Klein et al., 2005b). Our activity in the field of stem cell transplantation has been maintained with reviews for Nature Neuroscience (Klein and Svendsen, 2005). Furthermore our group continues to be involved with the human GDNF trials for Parkinson's disease. Although a recent AMGEN double blind trial only showed modest improvement in outcome for their patients this is a highly controversial study that used a very different catheter system to

the one used in our initial trial (Gill et al., 2003). We have recently published an update paper showing that all of our patients continue to do very well following 2 years of GDNF delivery with little sign of any serious side effects (Patel et al., 2005b). Therefore we remain committed to GDNF for Parkinson's disease and still feel that cellular delivery using the methods outlined above will prove to be the easiest and safest method of choice for veterans and the general public alike. We are currently applying to the FDA for a clinical trial using similar cells for transplantation into ALS patients. This will lay the groundwork for moving the current study into the clinic for Parkinson's disease.

Conclusions

We feel we have been making great strides in accomplishing our proposed goals for the second year of the grant. We are continuing to assess the survival and therapeutic potential of hNSC secreting GDNF in rodent and primate models of PD. We are also trying to determine the optimal transplantation condition for the hNSC, including what pre-treatment of the cells is needed, the optimal number of cells transplanted, and the most effective transplantation sites into the rat brain. Our goals for the third year are to determine GDNF regulation in vivo, test therapeutic benefits of GDNF in a rodent model of PD, and use PET imaging to monitor effect of GDNF regulation on the dopamine system. We also aim to determine the amount of GDNF needed to be effective and how long therapeutic benefits will remain if GDNF expression is turned off in vivo.

Reference List

- Deglon N, Tseng JL, Bensadoun JC, Zurn AD, Arsenijevic Y, Pereira dA, Zufferey R, Trono D, Aebischer P (2000) Self-inactivating lentiviral vectors with enhanced transgene expression as potential gene transfer system in Parkinson's disease. *Hum Gene Ther* 11:179-190.
- Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, Brooks DJ, Svendsen CN, Heywood P (2003) Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat Med* 9:589-595.
- Klein S, Svendsen CN (2005) Stem cells in the injured spinal cord: reducing the pain and increasing the gain. *Nat Neurosci* 8:259-260.
- Klein SM, Behrstock S, McHugh J, Hoffmann K, Wallace K, Suzuki M, Aebischer P, Svendsen CN (2005) GDNF Delivery Using Human Neural Progenitor Cells in a Rat Model of ALS. *Hum Gene Ther* 16:509-521.
- Matsuura K, Makino H, Ogawa N (1997) Cyclosporin A Attenuates the Decrease in Tyrosine Hydroxylase Immunoreactivity in Nigrostriatal Dopaminergic Neurons and in Striatal Dopamine Content in Rats with Intrastriatal Injection of 6-Hydroxydopamine. *Experimental Neurology* 146:526-535.
- Patel NK, Bunnage M, Plaha P, Svendsen CN, Heywood P, Gill SS (2005) Intraputamenal infusion of glial cell line-derived neurotrophic factor in PD: a two-year outcome study. *Ann Neurol* 57:298-302.
- Zufferey R, Donello JE, Trono D, Hope TJ (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* 73:2886-2892.

APPENDIX 1:

**Human neural progenitors deliver functional glial derived
neurotrophic factor (GDNF) to parkinsonian rodents and aged
primates**

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Abstract

Glial cell line-derived neurotrophic factor (GDNF) has been shown to increase the survival and functioning of dopamine neurons in a variety of animal models and some recent human trials. However, delivery of such a large protein to the brain remains a challenge. Here we show that human neural progenitor cells (hNPC) can be genetically modified to release glycosylated GDNF *in vitro* under an inducible promoter system. hNPC^{GDNF} were then transplanted into the striatum of rats ten days following a partial lesion of the dopamine system. Two weeks following transplantation the cells had migrated within the striatum and were releasing physiologically relevant levels of GDNF. This was sufficient to increase host dopamine neuron survival and fiber outgrowth. At five weeks following grafting there was functional improvement in transplanted animals and at eight weeks the cells had migrated to fill most of the striatum and continued to release GDNF with transport to the substantia nigra. These cells could also survive and release GDNF three months following transplantation into the aged monkey brain. No tumors were found in any animal. Human neural progenitor cells can be genetically modified, and thereby represent a novel, safe and powerful option for delivering growth factors to specific targets within the central nervous system. GDNF released at functional levels by these cells may be relevant to neurological diseases, such as Parkinson's disease and ALS and aging.

Introduction

Glial cell line-derived neurotrophic factor (GDNF) was first isolated by virtue of its neuroprotective and trophic effects on dopamine neurons that are lost in Parkinson's disease (PD); (Lin et al., 1993). Although GDNF does not penetrate the brain from the blood, studies in Parkinsonian primates showed that direct infusion into the ventricles led to a reversal of symptoms (Gash et al., 1996). Furthermore, a series of experiments in both monkeys and rats have shown that GDNF can prevent dopamine neuron cell loss and induce fiber sprouting in a wide range of different models of PD (Bjorklund et al., 1997). This led to a clinical trial where GDNF was administered via bolus injections directly into the ventricles of Parkinson's disease patients. The outcome of this trial was negative, with some significant side effects reported (Nutt et al., 2003; Kordower et al., 1999). This may have been due to poor penetrance of GDNF from the ventricles into the deep posterior regions of the putamen affected in PD. In order to overcome this problem, GDNF has now been continually infused using pumps and catheters directly into the caudal putamen of patients with PD over a period of 3 years. The results of this open label study showed that GDNF was safe and provided significant decreases in Parkinsonian symptoms and significant increases in dopamine storage within the brain (Gill et al., 2003; Patel et al., 2005a). However, delivery of proteins

via a catheter and pump is complex, the GDNF concentrations have to be very high, the pumps need regular refilling and delivery is only to a point source within the putamen.

Direct gene therapy using live virus has emerged as one alternative method of growth factor delivery, and has been shown to work well in both rodent and primate models of PD (Choi-Lundberg et al., 1997; Kordower et al., 2000; Bjorklund et al., 2000). However, there are risks that transport of live virus to ectopic regions of the CNS will produce side effects (Hsich et al., 2002). Furthermore, the possibility of viral leakage to the blood system and integration into actively transcribed sites involved with cellular transformation remain an issue (Hacein-Bey-Abina et al., 2003). Modification of cells in the culture dish and subsequent transplantation is termed *ex vivo* gene therapy where no live virus is injected into the subject, thus increasing safety. Autologous fibroblasts are an obvious choice for *ex vivo* gene therapy. They can be modified in the dish to release growth factors, survive transplantation and survive for extended periods in the brain (for review see (Gage, 1998). Recently, this technique has been used to deliver NGF to the basal forebrain of 10 patients with Alzheimer's disease (Blesch and Tuszynski, 2004). While the use of autologous cells has some benefit, it is not essential as the human brain can accept allografts well following only brief suppression based on fetal transplant studies in PD (Lindvall, 1994). Furthermore, fibroblasts do not migrate following transplantation and so drug delivery is limited to only the immediate site of the transplant.

Human neural progenitor cells (hNPC) react very differently to either fibroblasts or primary fetal tissue following transplantation. Rather than forming a transplant core of non-migrating cells, a single deposit can fill almost the entire striatum (Svendsen et al., 1997; McBride et al., 2004). Rodent neural progenitor cells can also be genetically modified to produce GDNF that is physiologically active on dopamine neurons, both *in vitro* and following transplantation (Ostenfeld et al., 2002). In addition, a mouse immortal cell line has been generated that produces GDNF, survives transplantation in rodent models of PD and can prevent degeneration of dopamine neurons when transplanted before the lesion (Akerud et al., 2001; Ourednik et al., 2002). However, normal rat cells or immortalized mouse cells are not appropriate for human clinical trials which will require human cells. Furthermore, it will be crucial to demonstrate efficiency of the cells after the lesion rather than before to be relevant to PD.

Recently, efficient methods have been developed for the growth and differentiation of well characterized human neural progenitor cells. These can be isolated from post-mortem fetal brain tissue and expanded for significant periods of time in culture (Svendsen et al., 1998; Wright et al., 2003). A number of reports have shown that hNPC can differentiate into both astrocytes and neurons following transplantation into

the nervous system (Svendsen et al., 1997; Vescovi et al., 1999; Svendsen et al., 1998; Fricker et al., 1999; Flax et al., 1998; Englund et al., 2002) and lead to functional restoration in a model of Huntington's disease through neuroprotective effects on dying striatal neurons (McBride et al., 2004). However, naïve hNPC do not have any significant functional effects in models of PD (Burnstein et al., 2004). We have shown that hNPC engineered to produce GDNF and transplanted into the spinal cord can survive and functionally protect motoneurons in a rodent model of amyotrophic lateral sclerosis (Klein et al., 2005a). Here we use hNPC modified to release GDNF as a unique delivery option for trophic factor to the parkinsonian rat and aged monkey brain.

Results

Human neural progenitor cells can be modified to release GDNF under a regulated promoter system

The ^{ind}lentiGFP construct (Fig. 1a) was used to optimize lentiviral infection of hNPC and subsequent gene regulation. Small, intact spheres (<200µm) were co-infected with lenti-tTA and ^{ind}lentiGFP resulting in a high percentage of GFP-expressing cells (Fig. 1b). In the presence of doxycycline for 48 hours, GFP was almost entirely shut-off (Fig 1b). Robust GFP expression returned when doxycycline was removed for 48 hours, suggesting that tight and reversible regulation of this marker gene could be achieved (Fig 1b). Lentiviral infection of hNPC did not affect proliferation, shown by increased neurosphere size in phase, or subsequent differentiation into astrocytes and neurons (data not shown).

Having optimized lentiviral infection and regulation of human neural cells using the visible GFP reporter, neurospheres were next co-infected with the lenti-tTA and ^{ind}lentiGDNF constructs (Fig 1a).

Neurospheres infected with ^{ind}lenti-GFP did not release GDNF at levels measurable even with sensitive detection methods like gene chip analysis for wild type hNPC (Wright et al., 2003) and ELISA for rodent neurospheres (Ostenfeld et al., 2002). Neurospheres infected with the ^{ind}lentiGDNF construct (hNPC^{GDNF}) produced large amounts of glycosylated GDNF revealed by Western blot analysis (Fig. 1c; lane 3). This naturally glycosylated GDNF was similar to the glycosylated form produced by a non-human mammalian cell line (Fig 1c; lane 2) and was in contrast to the non-glycosylated human recombinant GDNF produced by bacteria (Fig. 1c, lane 1) and used for previous clinical trials (Gill et al., 2003). Immunocytochemistry for GDNF showed that over 40% of the hNPC following infection expressed GDNF while other cells were completely negative following acute neurosphere dissociation and plating for 1 hour (Fig. 1d). The protein was seen to accumulate within cells and had a punctate appearance consistent with normal production in golgi and release into the medium (Fig. 1d). Addition

of doxycycline to the media significantly reduced GDNF levels overtime to approximately 10% of "on" values without doxycycline by 8 days (Fig. 1e). The reason for this slow and incomplete shut-off in GDNF protein levels when compared to GFP may be related to the long half-life of GDNF mRNA. hNPC^{GDNF} plated for 8 days were shown to release approximately 8-10ng GDNF/ microgram of protein into the medium over a 24 hour period (Fig 1f). GDNF release could be switched off by doxycycline treatment and on again by removing doxycycline from the medium for 8 days (Fig 1f), showing that the regulation was reversible. GDNF-transduced hNPC remained healthy, continued to expand and produced stable amounts of GDNF for at least 20 weeks in culture. Thus, hNPC can be modified to secrete naturally glycosylated and regulated human GDNF for long periods *in vitro*.

GDNF release from hNPC is physiologically active *in vitro*

Having shown that hNPC^{GDNF} release high levels of the protein, we proceeded to establish possible functional effects *in vitro*. Primary rat dopamine neurons were cultured in either basal media, supernatant from hNPC or supernatant from hNPC^{GDNF}. The dopamine neurons were grown under minimal culture conditions to induce stress leading to low survival rates. The number of primary neurons staining for tyrosine hydroxylase (TH), the rate limiting enzyme for dopamine production, significantly increased when media from wild type human neurospheres was added one hour after plating, suggesting a protective effect from secreted factors produced by the hNPC ($p < .001$; Fig 2a,c) and supporting our earlier observations that neural stem cells alone can increase the survival of primary dopamine neurons (Ostenfeld et al., 1999). Supernatant from hNPC^{GDNF} had comparable effects to that produced from wild type hNPC on overall dopamine neuron number (Fig. 2c). In contrast, conditioned supernatant from hNPC^{GDNF} significantly increased both dopamine neuron neurite outgrowth and cell body area when compared with supernatant from wild type hNPC ($p < .001$; Figs. 2b, d, and e). Together these data show that while conditioned media from human neural cells is protective for dopamine neurons, only human neural progenitor cells modified to release GDNF can achieve powerful trophic effects on dopamine fiber outgrowth and cell body area.

hNPC^{GDNF} induce dopamine fiber outgrowth two weeks following transplantation into the partially 6-OHDA lesioned rat brain.

In order to establish whether the hNPC^{GDNF} could survive for short time periods following transplantation and modulate dopaminergic functioning, we grafted 120,000 cells into the striatum of rats with unilateral partial terminal lesions of the dopaminergic system. In this partial lesion, 6OHDA injected into the striatum selectively damages a proportion of dopaminergic fibers modeling the expected situation in the human striatum of a patient with mid-stage PD (Kirik et al., 1998). The results are described in Fig. 3a.

Animals had a reproducible partial lesion damage evidenced by loss of TH expression within the lesion core and penumbra and intact expression in distal/surrounding regions. hNPC^{GDNF}, survived transplantation into the core of the lesion and were detected using a human nuclei specific antibody (hNuc). GDNF staining revealed a good correlation between surviving hNPC^{GDNF} stained with hNuc marker and GDNF expression, although there was some diffusion of GDNF away from the graft core. There was no GDNF staining in animals receiving wild type hNPC transplants nor on the non-lesioned side of the brain, demonstrating the selective release of GDNF from engineered cells and the specificity of this technique.

In every animal, GDNF-expressing hNPC induced sprouting of TH-positive fibers at two weeks post-transplantation. In contrast, animals receiving wild type hNPC showed no TH fiber sprouting suggesting that the effect was due to GDNF release from the hNPC and not simply the cells alone. Cell counts of the total number of dopamine neurons surviving in each group revealed significantly more following hNPC^{GDNF} transplantation (Fig. 3b). GDNF is known to be retrogradely transported from terminals in the striatum back to the mesencephalon (Ai et al., 2003). Animals with hNPC^{GDNF} transplants in the striatum showed GDNF expression in dopamine neurons within the mesencephalon (Fig. 3c-e), demonstrating that GDNF released in the striatum was taken up by dopaminergic terminals and retrogradely transported to dopaminergic cell bodies.

We next asked whether GDNF released from the hNPC could be regulated *in vivo*. Transplanted animals were treated for 14 days with doxycycline but we saw little down regulation of GDNF immunoreactivity in the grafts (data not shown). This is probably due to the high sensitivity of the GDNF antibody and incomplete down regulation of GDNF expression in this system as shown *in vitro* (Fig 1e). Thus, further modifications to the viral construct may be required to reduce GDNF *in vivo* expression levels below detection.

Long-term survival and expression of hNPC^{GDNF} in the rat and primate brain

To establish whether the GDNF-expressing hNPC could induce functional recovery in this model of PD, a larger group of animals were transplanted with 240,000 cells over two sites and tested for apomorphine-induced rotation before and after transplantation (Fig 4a). Control (non-transplanted) animals showed significant spontaneous recovery over time (Fig. 4b) in this partial terminal lesion model of PD. We attribute this to the daily cyclosporine treatment received by all animals which is known to lead to sprouting and protection of dopamine neurons in this model (Matsuura et al., 1997). Notably, the group with hNPC^{GDNF} transplants recovered faster over the first 6 weeks of testing (Fig 4b) although this did not

reach significance when compared over the whole 10 week time course using ANOVA. Even though all animals showed functional recovery, there were significantly more dopamine neurons in the hNPC^{GDNF} transplant group (Fig. 4c) suggesting a survival promoting effect even at this late time stage.

Anatomical analysis revealed that hNPC^{GDNF} survived up to 10 weeks following transplantation and migrated away from the transplant to fill a large area of the striatum (Fig. 4d). Individual host neurons could be seen in the striatum decorated with GDNF based on immunocytochemical staining (Fig. 4e). Further immunohistochemistry revealed an overlap between hNPC^{GDNF} and GDNF expression, with substantial release of GDNF covering the striatum in all transplanted animals (Fig. 4f). Interestingly, there was no increase in TH expression in these long term animals in regions of high GDNF activity and dense cell transplants (data not shown). This may be due to a down regulation of TH in response to GDNF over long but not short time periods shown in a number of previous studies (Georgievska et al., 2002).

The graft anatomy clearly demonstrated that hNPC^{GDNF} had good survival and robust GDNF expression for a long period in the Parkinsonian lesioned brain. We next wanted to establish what types of cells had been generated. Double labeling of the sections with an antibody to glial fibrillary acidic protein (GFAP; astrocyte marker) or nestin (progenitor marker) along with a human nuclear antigen (hNUC; human specific marker) revealed that very few of the cells that migrated into the striatum had matured into astrocytes at this time point (<5% GFAP positive; Fig. 5A). A rare example is shown in Fig. 5b,c and d. Instead, the majority of the migrating cells continued to express nestin (>90% nestin positive; Fig. 5C). None of the cells were seen to differentiate into neurons using NeuN staining in combination with hNUC (data not shown).

In a small pilot study three aged rhesus monkeys were transplanted with hNPC^{GDNF} into the putamen. One animal died due to cyclosporine complications, one showed no transplant perhaps due to rejection but the last animal had a robust transplant that expressed high levels of GDNF three months after grafting (Fig 6). This shows that the cells used in this study are able to survive and release GDNF in the primate brain for extended periods of time.

Discussion

Since the discovery of nerve growth factor (Levi-Montalcini and Angeletti, 1963) there has been an intense interest in the potential of growth factors for restoring the damaged central nervous system. GDNF has been a focus due to its potent effects on dopamine neurons (Lin et al., 1993), although it has

since been found to bind to receptors and exert trophic effects on a variety of neurons within the brain (Airaksinen and Saarma,). As late-stage Parkinson's disease attacks a range of neural transmitter systems in addition to dopamine, this pleiotrophic aspect of GDNF may benefit a wider range of neurons leading to enhanced effects in patients. However, delivery of GDNF has been a consistent challenge. A few studies have used immortal cell lines to deliver GDNF before the lesion and shown protection (Akerud et al., 2001). Here we show for the first time that human genetically modified human neural progenitor cells can act as long-term "mini pumps" to deliver GDNF to specific regions of the rodent and primate brain and have effects after the lesion had been administered.

Engineered GDNF-secreting hNPC were remarkably stable over multiple passages and consistently released the same level of glycosylated GDNF into the medium. This allows banking and full characterization of the cells prior to transplantation. Clearly, regulation of GDNF release would be of interest from both a safety and dosing perspective for clinical trials. Inducible promoter systems driving tyrosine hydroxylase expression packaged in adenoviral vectors have been shown to work with hNPC both *in vitro* and *in vivo* (Corti et al., 1999). We constructed a vector to attempt regulation of GDNF release following transplantation. While regulation *in vitro* was very efficient for the marker protein GFP,, we could not switch off GDNF production below/beyond approximately 10% of control values. *In vivo* we were not able to detect down-regulation of GDNF in response to doxycycline. This result is similar to other reports where inducible vectors have been used to control the release of GDNF following direct injection of viral vectors to the brain, but only met with limited success (Georgievska et al., 2004) and may be the result of an especially long half-life for GDNF RNA compared to TH or GFP combined with very sensitive methods of detection. We are currently exploring this issue further, and developing single vector systems to improve the regulated production of GDNF.

The safety of progenitor cell transplants is always a key concern. Neural tissues derived from mouse embryonic stem cells can form teratomas in the brain (Bjorklund et al., 2002). In contrast, the human progenitor cells used in the current study are derived from fetal brain tissue not human embryonic stem cells. Fetal derived human neural progenitor cells have been transplanted in hundreds of studies and never been found to form tumors to date. We have previously shown that hNPC grown as neurospheres without dissociation during passage, do not express telomerase required for immortalization (Ostenfeld et al., 2000) and therefore have a finite lifespan in culture (Wright et al, in preparation). We have also shown that transplanted hNPC express Ki67 and divide *in vivo* for only a limited time (Ostenfeld et al., 2000). In the current study we confirm these observations as there was never any sign of overgrowth or tumor formation. Our unique method of hNPC propagation permits constant cell/cell contact and

eliminates exposure to serum or trypsin and stress from single cell dissociation (Svendsen et al., 1998). This technique may reduce the possibility of immortalization events associated with repetitive trypsin treatment and dissociation to single cells (cite nature biotech paper). In addition, hNPC grown as neurospheres are never exposed to mouse feeder layers reducing the possibility of contamination recently reported for human embryonic stem cells (Martin et al., 2005). Human neural progenitors can be, expanded to large numbers, genetically modified, tested for safety, banked, and may be more accepted after transplantation due to their human neural origin. Thus these cells represent an ideal vehicle for delivering proteins to the human brain.

Transplants of hNPC^{GDNF} survived well in the brain, released GDNF and increased the number of TH neurons and their fiber projections around the transplant site. Naïve hNPC had no effect on either survival or TH fiber sprouting, supporting our previous findings (Burnstein et al., 2004). This is in contrast to a study that suggested naïve immortalized mouse stem cells derived from the developing cerebellum (C17.2 cells) could restore dopamine neurons and function in a mouse model of PD (Ourednik et al., 2002). However, this may be due to the fact C17.2 cells were found to spontaneously release GDNF in contrast to the hNPC described in the current study. At six weeks following transplantation, animals with GDNF-expressing hNPC showed a trend towards functional effects when compared to control animals. By 10 weeks, this difference was no longer present due to spontaneous recovery in the control group likely related to the partial lesion and cyclosporin treatment. Many of the cells (>90%) maintained an immature phenotype as evidenced by constant nestin expression. This may reflect the long time course of maturation required for human progenitor cells when compared to rodent progenitors, or the lack of appropriate species specific differentiation signals in the rodent brain. In three aged monkeys hNPC^{GDNF} could survive and produce GDNF for up to 3 months showing that this method of delivery is also applicable to primates. Together these results show that combining progenitor cell therapy with *ex vivo* gene therapy is a powerful approach to the future treatment of PD.

Methods

Cell growth.

Human cortical neural progenitor cells were isolated from primary human fetal cortex between 10 and 15 weeks of gestation according to protocols approved by the NIH and local ethics committee at the University of Wisconsin Madison and by the University of Washington Birth Defects Laboratory. Following dissociation in 0.1% trypsin, cells were seeded at 200,000 cells per ml in T75 flasks containing DMEM/Ham's F12 (Gibco-BRL) supplemented with penicillin/streptomycin (Gibco-BRL, 1%), N2 (Gibco-

BRL, 1%), epidermal growth factor (Sigma, 20 ng/ml) with heparin (Sigma, 5 µg/ml) and fibroblast growth factor-2 (R&D, 20 ng/ml). Neurosphere colonies rapidly formed and were expanded by chopping using an automated tissue chopper as described in detail previously (Svendsen et al., 1998). At 10 weeks the cells were switched to medium containing LIF (Chemicon, 10 ng/ml) and under these conditions the cultures could be grown for at least another 30 weeks in a relatively stable form based on gene expression patterns and differentiation potential (Wright et al., 2003).

Lentiviral infection.

Our inducible lentiviral construct is based on the already published non-inducible one described in detail previously (Deglon et al., 2000) and is shown schematically in Figure 1. In this system, the mouse phosphoglycerate kinase 1 (PGK) promoter (strong constitutive promoter) drives the tTA1 in the lenti-tTA construct. The post-translational cis-acting regulatory element of the woodchuck hepatitis virus (WHV) is included and has been shown to significantly enhance transgene expression (Deglon et al., 2000). In the absence of doxycycline, tTA1 will bind to the tetO that is upstream of a minimal promoter driving the gene of interest (in this case GDNF in the indlenti-GDNF construct or GFP in the of indlenti-GFP construct). In the presence of doxycycline the tTA is unable to activate the transgene. The lentiviral particles were suspended in 1% fetal bovine serum albumin in phosphate buffered saline. Lentivirus infection was 12 hrs in culture medium with 25 ng of indlenti-GFP or indlenti-GDNF and 75 ng of lenti-tTA per 3 spheres.

Immunocytochemistry. Neurospheres infected with indlenti-GFP or indlenti-GDNF were dissociated using accutase (PAA Laboratories) and plated onto glass coverslips coated with poly-L-lysine (Sigma, 0.01%) and laminin (Sigma, 0.001%). Cells were plated at 30,000 per coverslip in differentiation media DMEM/Ham's F12 with 2% B27 (Gibco-BRL) for 7 days. Following fixation with 4% paraformaldehyde and block in 10% normal donkey serum, cells were stained for GDNF (goat, R&D 1:2000) with a tritc-conjugated secondary (Jackson Laboratories 1:1000) and hoechst.

GFP regulation.

Following indlenti-GFP infection, the GFP expression in a representative neurosphere was photographed, and a phase photograph was taken at the same time. This sphere was then cultured in media with doxycycline (Sigma, 100 ng/ml) for 48 hours and again photographed under both fluorescence and phase. Doxycycline was removed from the media for 48 hours. Following this washout, a photograph was again taken under both fluorescence and phase.

GDNF quantification and regulation.

Following indlenti-GDNF infection, GDNF levels and regulation were assessed. Neurospheres were dissociated with accutase and equally divided into wells. Wells (n=3) were maintained in B27 differentiation media and wells (n=3) were maintained in B27 differentiation media with doxycycline (1000 ng/ml). The plating media with or without doxycycline was collected every 2 days for 10 days and the samples were stored at -20°C for later analysis. GDNF was measured in the supernatant from indlenti-GDNF-infected neurospheres using a GDNF Elisa Kit (Promega), according to manufacturer's instructions. For each collection day, we report GDNF levels in the plus doxycycline groups as a percentage of the GDNF levels in the minus doxycycline groups. In addition to confirming GDNF shut-off overtime, we tested the reversible regulation of GDNF by measuring the supernatant from indlenti-GDNF infected cells plated in B27 differentiation media for 8 days without doxycycline, then with doxycycline for 8 days and finally following doxycycline washout for 8 days.

GDNF purification and western blotting.

Recombinant human GDNF (rhGDNF) was purified from indlenti-GDNF infected hNPC- media using heparin affinity chromatography. A 10 ml Heparin Sepharose (Amersham Biosciences, Uppsala, Sweden) column was equilibrated with 50 mM Na_2HPO_4 , pH 7.2. Two liters of NSC-GDNF conditioned media were loaded onto the column at a flow rate of 20 mL/min. The column was washed with three column volumes of equilibration buffer and the GDNF was eluted with approximately 1 column volume of 50 mM Na_2HPO_4 with 750 mM NaCl. The GDNF was collected in a 5 ml fraction and stored at -30°C .

The rhGDNF from the heparin sepharose fraction was concentrated by TCA precipitation. TCA (Sigma, St. Louis, MO) was added at a ratio of 1.0g TCA to 1.0 ml GDNF fraction. The precipitation proceeded at 4°C for 30 minutes before centrifugation at 13,000 rpm for 60 minutes. The precipitate was re-suspended in 1X LDS sample buffer (Invitrogen, Carlsbad, CA) for SDS-PAGE and Western Blot analysis.

Western Blot analysis was performed on partially purified rhGDNF from hNPC along with commercially available rhGDNF from the mouse mammalian cell line NSO (R&D Systems) and from E. coli (Peprotech). Samples diluted in LDS sample buffer and reducing agent were heated to 70°C for 5 minutes. SDS-PAGE was performed using NuPAGE®, 10% Bis-Tris gels (Invitrogen) GDNF was transferred to a PVDF membrane using the XCell SureLock™ and Blot Module. Western Blot was performed using the WesternBreeze® Immunodetection Chromogenic kit (Invitrogen). GDNF was detected with a goat anti-GDNF primary antibody.

GDNF functional effects in vitro.

Primary ventral mesencephalon was dissected from E14 embryos of Sprague-Dawley rats and plated onto poly-L-lysine (0.01%) and laminin (0.001%) coated coverslips. Cells were cultured for 7 days in either basal N2 (Gibco-BRL, 1%) media (n=3), supernatant from wild type neurospheres (n=3) or supernatant from neurospheres infected with indlenti-GDNF (n=3). Following fixation with 4% paraformaldehyde and rinses with phosphate buffered saline, cell cultures were stained for tyrosine hydroxylase (mouse, chemicon 1:200) with FITC-conjugated secondaries (Jackson Laboratories, 1:1000) and hoechst. Cells were viewed under a fluorescent microscope and four fields were analyzed from each of the 3 coverslips per group. Fluorescent digital images were captured with a digital video camera using the Spot camera image analysis system. The number of TH-positive cells was quantified by counting cells immunoreactive for TH in 12 randomly selected fields. The neurite length and cell body size was quantified by using metamorph to determine the μm and radius, respectively, for TH-positive cells in 12 randomly selected fields.

Partial 6-OHDA Lesion.

Adult male Lewis rats (300-350 g) were injected with a total of 21 μg of 6-OHDA resuspended in 0.9% saline with 0.2 μg ascorbic acid. Injections of 7 μg /3.5 μl were made over 3 sites from bregma: AP +1.0, ML -3.0, DV -5.0; AP -0.1, ML -3.7, DV -5.0; AP -1.2, ML -4.5, DV -5.0.

Cell Transplants.

Cells were plated as neurospheres for 1 week in B27 differentiation media supplemented with Ciliary Neurotrophic Factor. To prepare cells for transplantation, medium was removed, cells were rinsed, and accutase was added for 15 min at 37°C to detach plated spheres from T75 flask. Cells were collected, centrifuged at 1000 RPM for 5 minutes, accutase was removed and cells were rinsed. Cells were incubated in DNAase for 10 minutes at 37°C, centrifuged at 1000 RPM for 5 minutes, DNAase was removed and cells were dissociated in Liebovitz/0.6% glucose (1:1) supplemented with B27 (1:50). Cells were counted, centrifuged at 1000 RPM for 5 minutes, re-suspended at 66,500/ μl , and maintained on ice.

For the short-term (2 week) transplant experiments, hNPC (n=3) or hNPC^{GDNF} (n=3) were transplanted into one site at AP -0.3, ML -3.6, DV -5.2, one week post lesion. For the long-term (8 week) transplant experiments, hNPC^{GDNF} (n=9) were transplanted into two sites at AP +0.5, ML -3.3, DV -5.2 and AP -0.4, ML -3.7, DV -5.2, 10 days post lesion. Control animals (n=4) received lesion but no transplant. A 10

μl Hamilton syringe was lowered from dura, left in place for 1 minute before injecting 3 μl cells over 3 minutes, and retracted after an additional 3 minutes. Animals were injected with cyclosporin (i.p. 10 mg/kg) 1 day before and every day following transplantation. At 2 or 8 weeks post-transplantation animals were perfused with chilled 0.9% saline and 4% paraformaldehyde. Brains were cryoprotected in 30% sucrose with 0.1% sodium azide, and sectioned to 40 μm using a microtome.

Immunohistochemistry.

Sections were stained for human nuclei (Chemicon, mouse 1:20), GDNF (R&D, goat 1:250), tyrosine hydroxylase (TH, Chemicon, mouse), nestin (Chemicon, rabbit) and (glial fibrillary acidic protein (GFAP Chemicon). For human nuclei, sections were rinsed in Tris-HCl, incubated in 2N HCl for 30 minutes at 37°C, quenched in 5% H₂O₂ and 10% methanol, and blocked in 10% normal horse serum prior to primary antibody. Biotinylated mouse secondary (vector labs, 1:2000) and ABC (vectastain kit) were followed by DAB (Sigma tablets) development. For human GDNF, sections were rinsed in TBS-T, quenched in 0.1 M sodium periodate and blocked in 3% NHS and 2% BSA prior to primary antibody. Biotinylated goat secondary (vector labs, 1:200) and ABC were followed by DAB development with nickel ammonium sulfate enhancement. For TH staining, sections were rinsed in PBS, quenched in 5% H₂O₂ and 10% methanol, and blocked in serum. Tyrosine hydroxylase antibody was followed by biotinylated mouse secondary, ABC, and DAB for development. For labeling with nestin or double labeling with hnuclei and GFAP antibodies, sections were processed as for TH staining, but primary antibodies were followed by mouse TRITC- and rabbit FITC-conjugated secondaries (Jackson labs, 1:1000). For nigral sections double labeled with TH and GDNF, sections were processed for tyramide staining (kit) with mouse FITC conjugated secondary (Jackson labs) and goat TRITC conjugated secondary (Jackson labs).

GDNF functional effects in vivo.

TH positive neurons in the substantia nigra were viewed at 10x magnification under a light microscope. Images were captured with a digital video camera using the Spot image analysis system. At two and 8 weeks post-transplantation, TH positive neurons were quantified ipsilateral and contralateral to the lesion in three anatomically equivalent sections through the substantia nigra. TH positive neuron survival was calculated as percent of TH positive neurons remaining, and data are expressed as average percent TH neurons ± S.E.M (t-test).

Amphetamine-induced rotations (2.5 mgs/kg) were assessed one week following a partial 6-OHDA lesion and animals with >300 rotations/1.5 hour were separated into balanced transplantation groups. Cells

were transplanted 10 days following the lesion, and animals were retested weekly for amphetamine-induced rotations beginning five weeks post-transplantation. A trend was seen for recovery in animals receiving hNPC^{GDNF}, but this did not reach significance.

Monkey studies

Three aged monkeys (22,35,29 years of age) served as subjects. Coordinates for transplantation of GDNF stem cells were based upon MRI guidance. Under sterile conditions, monkeys received two injections of stem cells placed unilaterally into the caudate nucleus (10µl, 5µl) and three injections were placed into the putamen (10µl, 10µl 5µl) on the same side. One animal died from natural causes 2 months following transplantation. The remaining 2 monkeys were sacrificed 3 months following transplantation via perfusion with saline followed by fixation with a 4% Zamboni's fixative.

Figure legends

Figure 1. Tet-off lentiviral construct provides regulated GFP and GDNF production in hNPC. Cells were co-infected with SIN-PGK-tTA1 and either SIN-TRE-GDNF or SIN-TRE-GFP (a). Neurospheres infected with SIN-TRE-GFP show tight regulation of GFP by the addition and withdrawal of doxycycline (b). Western blot demonstrates GDNF production by hNPC infected with SIN-TRE-GDNF (lane 3). Molecular weight markers are on the left, hGDNF from mouse cell line (lane 1), rhGDNF from *E. coli* (lane 2) (c). Approximately 70% of cells were shown to express GDNF following infection with the inducible GDNF construct (d). GDNF levels in the presence of doxycycline compared to in the absence of doxycycline decrease following doxycycline treatment for 2 days and fall to over 90% shutdown in GDNF production following 10 days exposure (e). This regulation was dynamic as when doxycycline was removed for an 8 day washout, GDNF expression switched on again to levels similar to those seen before switch off (f).

Figure 2. Functional effects of GDNF released from hNPC *in vitro*. Following plating of primary rat dopamine neurons overall survival was significantly enhanced with either conditioned media from wild type hNPC or conditioned media from lenti-GDNF infected hNPC (c). However, when the fiber length (a, b, d) or cell body area (a, b, e) of dopamine neurons was assessed, significant increases were seen only in response to lenti-GDNF infected hNPC.

Figure 3. hNPC-GDNF survive in the partially lesioned striatum and protect dopamine neurons. Animals sacrificed at 2 weeks show GDNF release from hNSC-GDNF, but not wild-type hNPC, and show TH fiber sprouting around the graft site (a). GDNF released from hNSC-GDNF transplants in the striatum is

transported back to TH-expressing cell bodies in the substantia nigra (c-e) and increases the number of TH-positive cells (b).

Figure 4. hNPC-GDNF express GDNF long-term in the lesioned striatum and provide protection. Rats receiving hNPC-GDNF or no cells post-lesion were assessed for functional recovery by amphetamine rotations (a). Rats receiving hNPC-GDNF had a trend towards fewer rotations at 5 and 6 weeks post-transplantation compared to the control group. By 8 weeks, control rats showed similar recovery from the partial lesion (b). More TH-positive neurons are present in the hNPC-GDNF transplanted rats compared to no transplant rats (c). Cells survive for at least 8 weeks following transplantation (d) and release GDNF which is taken up by host neurons (e) and distributed to cover a large region of the striatum (f).

Figure 5. Differentiation of hNPC transplanted into the lesioned rodent striatum. Most of the cells migrating away from the core of the transplant did not stain for GFAP (a), although a few were clearly double labeled (b,c and d). The majority of migrating cells were stained for human nestin (e) which also co labeled with human nuclear protein (inset).

Figure 6. hNPC survive transplantation within the primate putamen three months after transplantation. Increasing magnification of the brain region containing a hNPC transplant stained for GDNF (a,b).

Reference List

Ai Y, Markesbery W, Zhang Z, Grondin R, Elseberry D, Gerhardt GA, Gash DM (2003) Intraputaminal infusion of GDNF in aged rhesus monkeys: distribution and dopaminergic effects. *J Comp Neurol* 461:250-261.

Airaksinen MS, Saarma M The GDNF family: signalling, biological functions and therapeutic value.

Akerud P, Canals JM, Snyder EY, Arenas E (2001) Neuroprotection through delivery of glial cell line-derived neurotrophic factor by neural stem cells in a mouse model of Parkinson's disease. *J Neurosci* 21:8108-8118.

Bjorklund A, Kirik D, Rosenblad C, Georgievska B, Lundberg C, Mandel RJ (2000) Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. *Brain Res* 886:82-98.

Bjorklund A, Rosenblad C, Winkler C, Kirik D (1997) Studies on neuroprotective and regenerative effects of GDNF in a partial lesion model of Parkinson's disease. *Neurobiol Dis* 4:186-200.

Bjorklund LM, Sanchez-Pernaute R, Chung S, Andersson T, Yin Ching Chen I, McNaught KP, Brownell A, Jenkins BG, Wahlestedt C, Kim K, Isacson O (2002) Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model.

Blesch A, Tuszynski MH (2004) Gene therapy and cell transplantation for Alzheimer's disease and spinal cord injury. *Yonsei Med J* 45 Suppl:28-31.

Burnstein RM, Foltynie T, He X, Menon DK, Svendsen CN, Caldwell MA (2004) Differentiation and migration of long term expanded human neural progenitors in a partial lesion model of Parkinson's disease. *Int J Biochem Cell Biol* 36:702-713.

Choi-Lundberg DL, Lin Q, Chang YN, Chiang YL, Hay CM, Mohajeri H, Davidson BL, Bohn MC (1997) Dopaminergic neurons protected from degeneration by GDNF gene therapy. *Science* 275:838-841.

Corti O, Sabate O, Horellou P, Colin P, Dumas S, Buchet D, Buc-Caron MH, Mallet J (1999) A single adenovirus vector mediates doxycycline-controlled expression of tyrosine hydroxylase in brain grafts of human neural progenitors. *Nat Biotechnol* 17:349-354.

Deglon N, Tseng JL, Bensadoun JC, Zurn AD, Arsenijevic Y, Pereira dA, Zufferey R, Trono D, Aebischer P (2000) Self-inactivating lentiviral vectors with enhanced transgene expression as potential gene transfer system in Parkinson's disease. *Hum Gene Ther* 11:179-190.

Englund U, Fricker-Gates RA, Lundberg C, Bjorklund A, Victorin K (2002) Transplantation of human neural progenitor cells into the neonatal rat brain: extensive migration and differentiation with long-distance axonal projections. *Exp Neurol* 173:1-21.

Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billingham LL, Jendoubi M, Sidman RL, Wolfe JH, Kim SU, Snyder EY (1998) Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. *Nat Biotechnol* 16:1033-1039.

Fricker RA, Carpenter MK, Winkler C, Greco C, Gates MA, Bjorklund A (1999) Site-specific migration and neuronal differentiation of human neural progenitor cells after transplantation in the adult rat brain. *J Neurosci* 19:5990-6005.

Gage FH (1998) Cell Therapy. *Nature* 392 (supplement):18-24.

Gash DM, Zhang Z, Ovadia A, Cass WA, Yi A, Simmerman L, Russell D, Martin D, Lapchak PA, Collins F, Hoffer BJ, Gerhardt GA (1996) Functional recovery in parkinsonian monkeys treated with GDNF. *Nature* 380:252-255.

Georgievska B, Jakobsson J, Persson E, Ericson C, Kirik D, Lundberg C (2004) Regulated delivery of glial cell line-derived neurotrophic factor into rat striatum, using a tetracycline-dependent lentiviral vector. *Hum Gene Ther* 15:934-944.

Georgievska B, Kirik D, Bjorklund A (2002) Aberrant sprouting and downregulation of tyrosine hydroxylase in lesioned nigrostriatal dopamine neurons induced by long-lasting overexpression of glial cell line derived neurotrophic factor in the striatum by lentiviral gene transfer. *Exp Neurol* 177:461-474.

Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, Brooks DJ, Svendsen CN, Heywood P (2003) Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat Med* 9:589-595.

Hacein-Bey-Abina S, et al. (2003) LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 302:415-419.

Hsieh G, Sena-Esteves M, Breakefield XO (2002) Critical issues in gene therapy for neurologic disease. *Hum Gene Ther* 13:579-604.

Kirik D, Rosenblad C, Bjorklund A (1998) Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-hydroxydopamine in the rat. *Exp Neurol* 152:259-277.

Klein S, Svendsen CN (2005) Stem cells in the injured spinal cord: reducing the pain and increasing the gain. *Nat Neurosci* 8:259-260.

Klein SM, Behrstock S, McHugh J, Hoffmann K, Wallace K, Suzuki M, Aebischer P, Svendsen CN (2005a) GDNF delivery using human neural progenitor cells in a rat model of ALS.

Klein SM, Behrstock S, McHugh J, Hoffmann K, Wallace K, Suzuki M, Aebischer P, Svendsen CN (2005b) GDNF Delivery Using Human Neural Progenitor Cells in a Rat Model of ALS. *Hum Gene Ther* 16:509-521.

Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, McBride J, Chen EY, Palfi S, Roitberg BZ, Brown WD, Holden JE, Pyzalski R, Taylor MD, Carvey P, Ling Z, Trono D, Hantraye P, Deglon N, Aebischer P (2000) Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science* 290:767-773.

Kordower JH, Palfi S, Chen EY, Ma SY, Sendera T, Cochran EJ, Mufson EJ, Penn R, Goetz CG, Comella CD (1999) Clinicopathological findings following intraventricular glial-derived neurotrophic factor treatment in a patient with Parkinson's disease. *Ann Neurol* 46:419-424.

Levi-Montalcini R, Angeletti PU (1963) Essentiality of nerve growth factor in the survival and maintenance of dissociated sensory and sympathetic embryonic nerve cells in vitro. *Developmental Biology* 7:653-659.

Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 260:1130-1132.

Lindvall O (1994) Neural transplants in Parkinson's disease. In: *Functional Neural Transplantation* (Dunnett SB, Björklund A, eds), pp 103-137. New York: Raven Press.

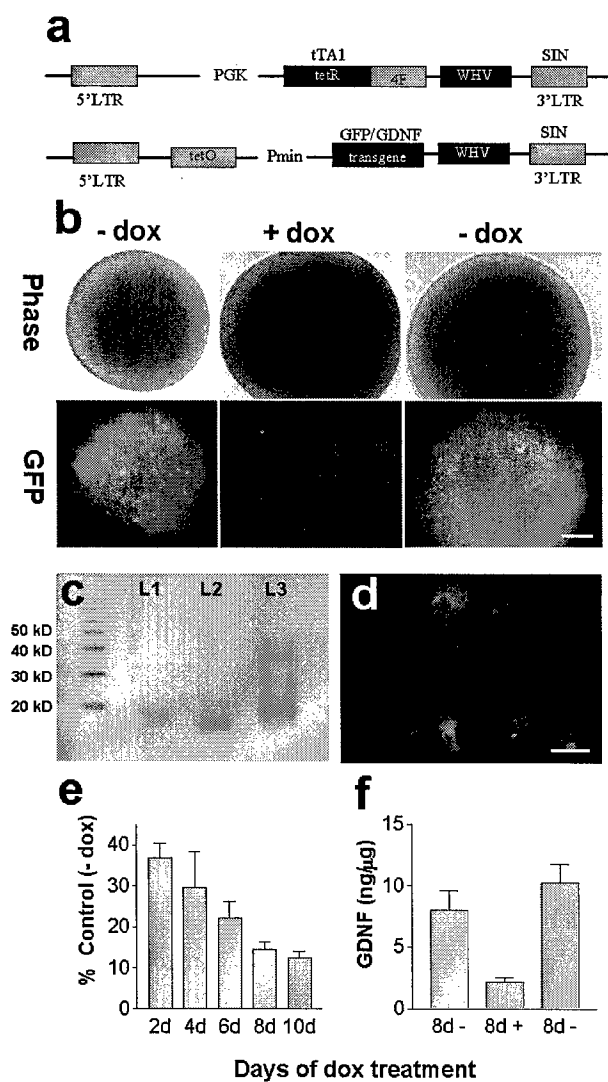
Martin MJ, Muotri A, Gage F, Varki A (2005) Human embryonic stem cells express an immunogenic nonhuman sialic acid. *Nat Med* 11:228-232.

Matsuura K, Makino H, Ogawa N (1997) Cyclosporin A Attenuates the Decrease in Tyrosine Hydroxylase Immunoreactivity in Nigrostriatal Dopaminergic Neurons and in Striatal Dopamine Content in Rats with Intrastriatal Injection of 6-Hydroxydopamine. *Experimental Neurology* 146:526-535.

McBride JL, Behrstock SP, Chen EY, Jakel RJ, Siegel I, Svendsen CN, Kordower JH (2004) Human neural stem cell transplants improve motor function in a rat model of Huntington's disease. *J Comp Neurol* 475:211-219.

- Nutt JG, Burchiel KJ, Comella CL, Jankovic J, Lang AE, Laws ER, Jr., Lozano AM, Penn RD, Simpson RK, Jr., Stacy M, Wooten GF (2003) Randomized, double-blind trial of glial cell line-derived neurotrophic factor (GDNF) in PD. *Neurology* 60:69-73.
- Ostenfeld T, Caldwell MA, Prowse KR, Linskens MH, Jauniaux E, Svendsen CN (2000) Human neural precursor cells express low levels of telomerase in vitro and show diminishing cell proliferation with extensive axonal outgrowth following transplantation. *Exp Neurol* 164:215-226.
- Ostenfeld T, Horn P, Aardal C, Orpen I, Caldwell MA, Svendsen CN (1999) Mouse epidermal growth factor-responsive neural precursor cells increase the survival and functional capacity of embryonic rat dopamine neurons in vitro. *NeuroReport* 10:1985-1992.
- Ostenfeld T, Tai YT, Martin P, Deglon N, Aebischer P, Svendsen CN (2002) Neurospheres modified to produce glial cell line-derived neurotrophic factor increase the survival of transplanted dopamine neurons. *J Neurosci Res* 69:955-965.
- Ourednik J, Ourednik V, Lynch WP, Schachner M, Snyder EY (2002) Neural stem cells display an inherent mechanism for rescuing dysfunctional neurons. *Nat Biotechnol* 20:1103-1110.
- Patel NK, Bunnage M, Plaha P, Svendsen CN, Heywood P, Gill SS (2005a) Intraputamenal infusion of glial cell line-derived neurotrophic factor in PD: A two-year outcome study. *Ann Neurol* 57:298-302.
- Patel NK, Bunnage M, Plaha P, Svendsen CN, Heywood P, Gill SS (2005b) Intraputamenal infusion of glial cell line-derived neurotrophic factor in PD: a two-year outcome study. *Ann Neurol* 57:298-302.
- Svendsen CN, Caldwell MA, Shen J, ter Borg MG, Rosser AE, Tyers P, Karmiol S, Dunnett SB (1997) Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. *Exp Neurol* 148:135-146.
- Svendsen CN, ter Borg MG, Armstrong RJ, Rosser AE, Chandran S, Ostenfeld T, Caldwell MA (1998) A new method for the rapid and long term growth of human neural precursor cells. *J Neurosci Methods* 85:141-152.
- Vescovi AL, Parati EA, Gritti A, Poulin P, Ferrario M, Wanke E, Frolichsthal-Schoeller P, Cova L, Arcellana-Panlilio M, Colombo A, Galli R (1999) Isolation and cloning of multipotential stem cells from the embryonic human CNS and establishment of transplantable human neural stem cell lines by epigenetic stimulation. *Exp Neurol* 156:71-83.
- Wright LS, Li J, Caldwell MA, Wallace K, Johnson JA, Svendsen CN (2003) Gene expression in human neural stem cells: effects of leukemia inhibitory factor. *J Neurochem* 86:179-195.
- Zufferey R, Donello JE, Trono D, Hope TJ (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* 73:2886-2892.

Figure 1



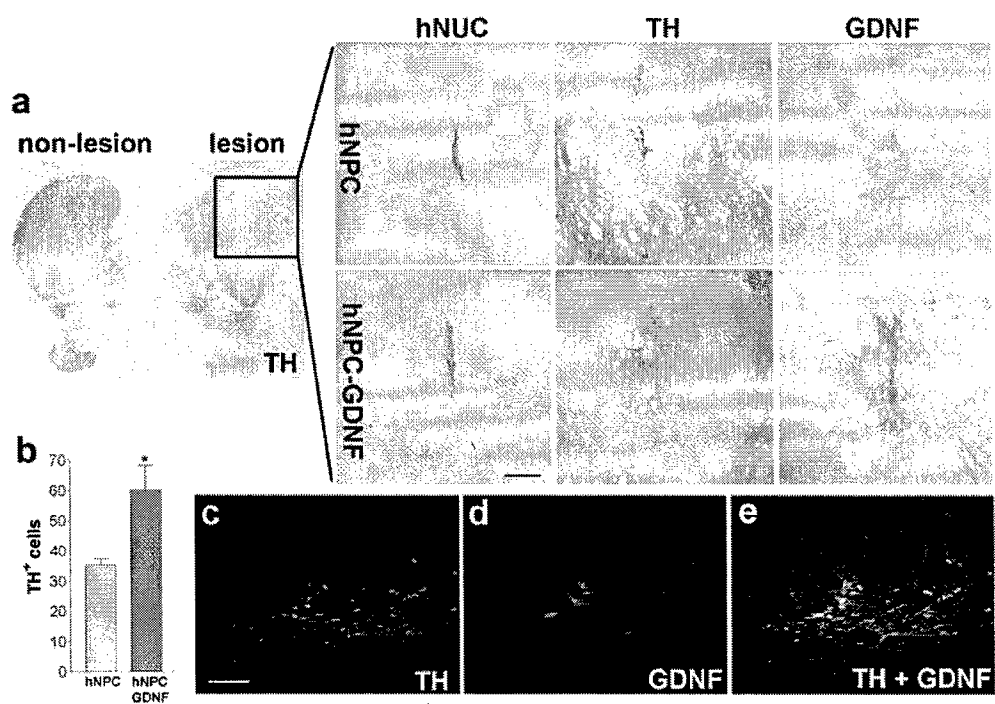


Figure 2

Figure 3

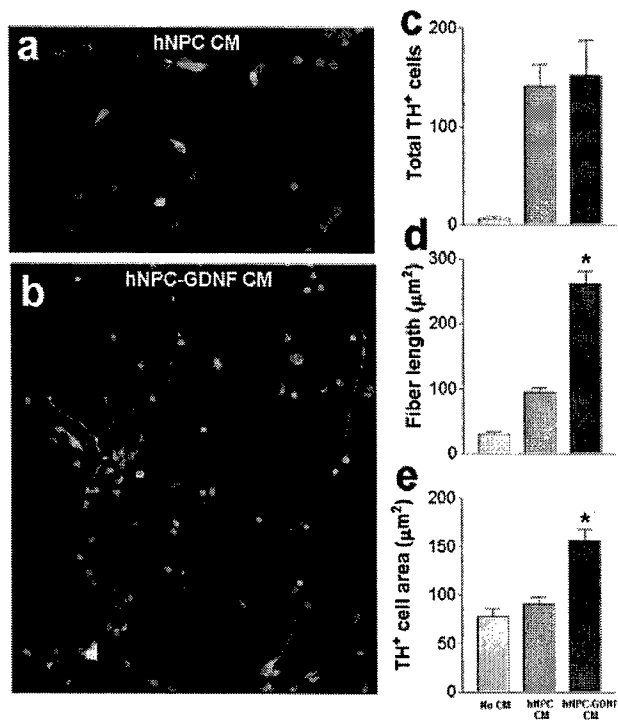


Figure 4

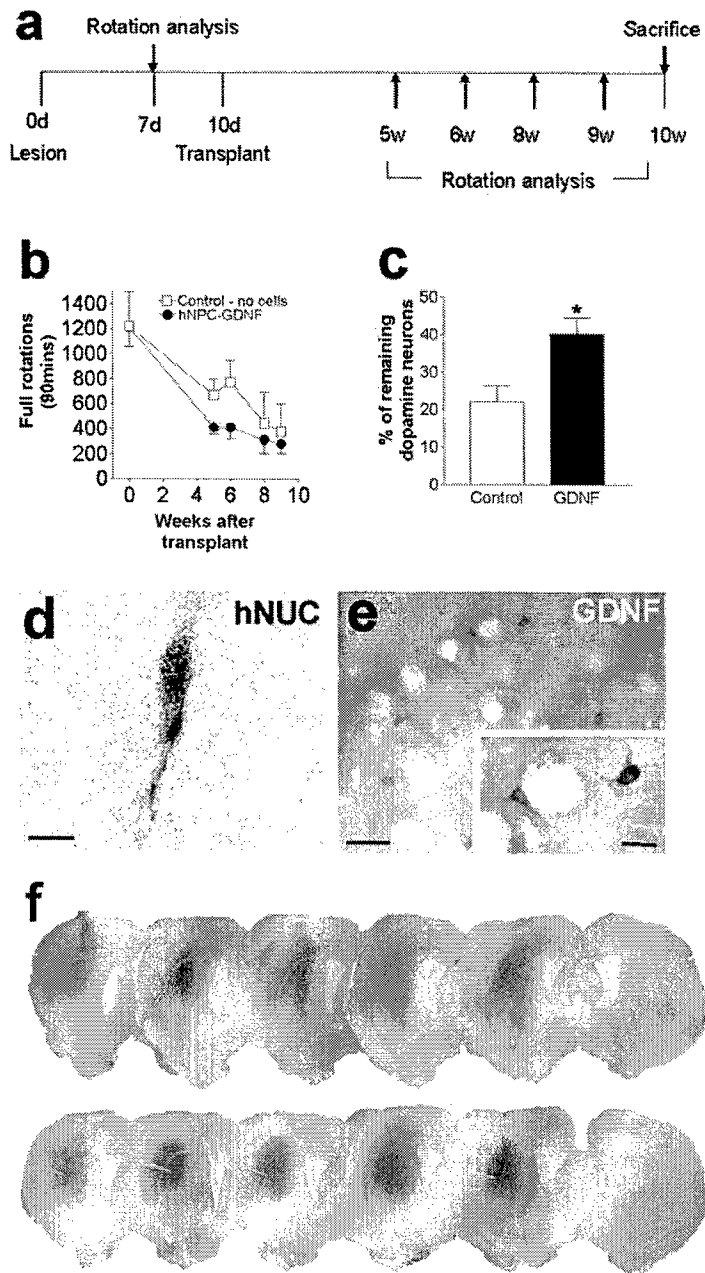


Figure 5

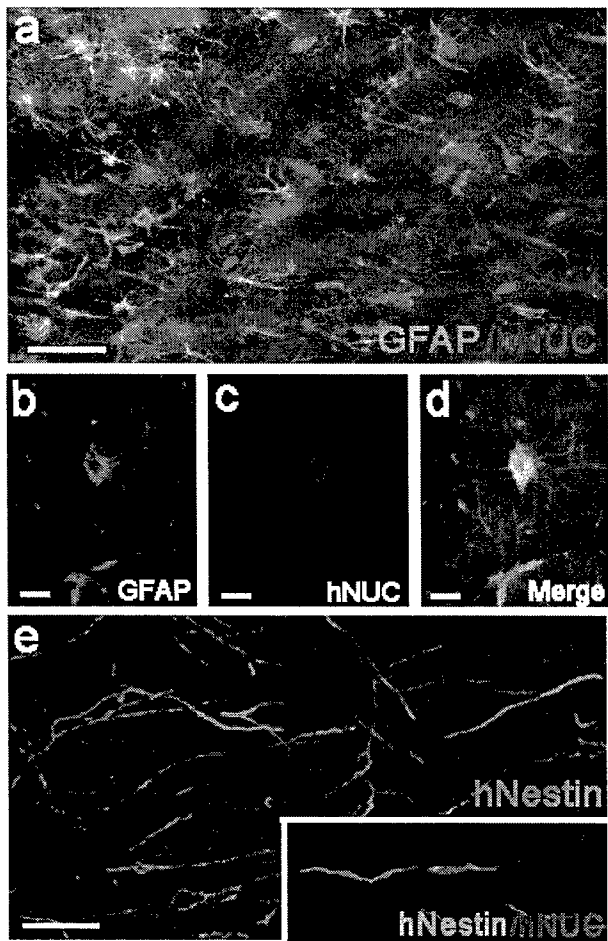


Figure 6

